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## Enrichment and characterization of thymus-repopulating cells in stroma-dependent cultures of rat bone marrow

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### SUMMARY

The bone marrow precursor cells seeding the thymus have been difficult to investigate using fresh bone marrow and *in vivo* thymus reconstitution assays. We have therefore designed a short-term bone marrow culture system allowing the study of thymus-repopulating cells in the marrow microenvironment. Low-density rat bone marrow cells were grown on pre-established mouse bone marrow stromal cell layers. Cocultured cells were maintained either under steroid-free conditions (Whitlock/Witte-type culture) or in the presence of  $10^{-7}$  M hydrocortisone (Dexter-type culture). After 3 days *in vitro*, the unanchored cell fractions were tested for their ability to colonize and repopulate fetal mouse thymic lobes *in vitro*. Both fresh low-density cells and Whitlock/Witte-type cultures, but not Dexter-type cultures, gave rise intrathymically to significant numbers of rat donor-type Thy-1.1<sup>high</sup> CD2<sup>+</sup> CD5<sup>low</sup> CD43<sup>+</sup> cells accounting for 50% to 90% of the organ-cultured cells at day 14. Repopulation of fetal mouse thymic lobes by rat Thy-1.1<sup>high</sup> cells could be used as a readout assay for initiation of thymopoiesis from bone marrow pre-

cursor cells, since 90% of the cells were CD3<sup>-low</sup> and TCR $\alpha\beta$ <sup>-low</sup> and 15% of the cells co-expressed CD4 and CD8. Dose-response analysis showed that thymus repopulating cells were at least maintained, if not amplified during the 3-day culture period, leading to at least a 10-fold enrichment as compared to unfractionated bone marrow. Unlike fresh low-density cells before culture, short-term Whitlock/Witte-type cultures were depleted in myeloid-restricted precursor cells. In culture, the thymus-repopulating activity was predominantly associated with a 10% lymphoid cell subset which did not express the B-lineage-associated antigens revealed by HIS24 (the rat B220 equivalent) and HIS50 mAbs. We propose that unanchored thymus-repopulating cells enriched in Whitlock/Witte-type cultures may represent lymphoid-restricted, T-cell precursors of the bone marrow capable of emigrating and colonizing the thymus.

Key words: T-cell development, fetal thymic organ culture, haemopoiesis, bone marrow cultures

### INTRODUCTION

During postnatal life in mammals, the bone marrow is the major source of T-cell precursors capable of colonizing and repopulating the thymus (Katsura et al., 1988). In addition, it is the primary site for the development of erythroid, myeloid and B-cell lineages, all of which derive from pluripotent stem cells (Abramson et al., 1977; Dick et al., 1985; Keller et al., 1985). In the past few years, we established that a chemotactic mechanism contributes to the recruitment of T-cell precursors to the thymus both in rats and chicken (Deugnier et al., 1989; Dargemont et al., 1989; Dunon et al., 1990). However, the nature of the precursor cells seeding the thymus is still uncertain.

The early stages of T-lineage differentiation have been difficult to investigate using fresh bone marrow. The frequency of bone marrow-resident T-cell precursors capable

of repopulating the thymus after intrathymic transfer has been estimated to be one in  $10^4$  nucleated cells (Katsura et al., 1988; Goldschneider et al., 1986; Spangrude et al., 1988). Moreover, T-cell precursors are phenotypically heterogeneous, since they have been found in two rare cell subsets of mouse bone marrow, both characterized by low levels of Thy-1 expression but discriminated by the absence (Lin<sup>-</sup>) or presence (Lin<sup>+</sup>) of lineage-specific surface antigens such as B-lineage-associated B220 antigens (Spangrude et al., 1988). Among these cell subsets, the Thy-1<sup>low</sup> Sca-1<sup>+</sup> Lin<sup>-</sup> cell subpopulation is particularly enriched in thymus-repopulating activity. However, this phenotypically homogeneous cell subset is functionally heterogeneous since it is similarly enriched in long-term, hematopoietic repopulating cells, in early B-cell precursors and in day 12 splenic colony-forming units. Thus, it is still not clear whether this precursor cell subset is mainly composed of marrow-repop-

ulating cells, or also contains thymus-homing cells, which may include lymphoid-restricted precursor cells (Wu et al., 1991; Ikuta et al., 1992).

An alternative approach to studying the early stages of T-cell development that has been little explored, consists of analyzing T-cell precursors in their marrow microenvironment by means of stroma-dependent bone marrow cultures. Long-term cultures of bone marrow, first introduced by Dexter and colleagues (Dexter et al., 1984), have proved to be extremely important model systems for studies on the regulation of myeloid and B-lymphocyte production (Dorshkind, 1990; Kincade et al., 1989). Dexter-type cultures, grown in the presence of corticosteroids, favour the development of differentiated myeloid cells from their precursors while simultaneously allowing precursor cells with long-term T- and B-cell differentiation ability to persist mainly as stroma-associated cells (Dexter et al., 1984; Fulop and Phillips, 1989). In contrast, corticosteroid-depleted culture conditions described by Whitlock and Witte (1982) are permissive for B-lymphopoiesis, as assessed by the production of unanchored sIgM<sup>+</sup> immature B-cells. Whitlock/Witte-type cultures of mouse bone marrow also contain cells capable of reconstituting T-lymphoid functions in recipient SCID (severe combined immunodeficiency) mice (Dorshkind et al., 1986). In both cell culture systems, the reconstituting cells have not been characterized and most importantly, their thymus-homing ability remains unknown.

We postulated that the production of thymus-homing cells in stroma-dependent bone marrow cultures could be regulated in a way which is similar to myeloid and B-lymphoid cell production, with sequential steps of adhesion and de-adhesion to the stroma leading to the release of lymphoid-restricted cells capable of colonizing the thymus. Accordingly, we investigated the presence of thymus-repopulating cells in the unanchored fraction of short-term rat bone marrow cultures grown on a preestablished stroma. Whitlock/Witte-type cultures, but not Dexter-type cultures, contained rat bone marrow cells capable of repopulating mouse fetal thymic lobes and initiating thymopoiesis *in vitro*. These short-term cultures were enriched in thymus-repopulating activity as compared to unseparated fresh bone marrow, but were depleted in myeloid-restricted precursor cells. Thus, unanchored steroid-sensitive, thymus-repopulating cells may fulfil the criteria for lymphoid-restricted cells which are capable of emigrating from the bone marrow before homing to the thymus.

## MATERIALS AND METHODS

### Animals

Pregnant female Thy-1.2 Swiss-derived and Balb/c mice, male Thy-1.2 Swiss-derived and Balb/c mice, and male Thy-1.1 WAG Wistar rats were obtained from animal facilities of the Centre National de la Recherche Scientifique (Villejuif, France) and IFFA-credo (L'Arbresle, France). Mouse embryos were dissected at day 15 of gestation, the appearance of vaginal plugs being designated as day 0.

### Short-term cultures of bone marrow

Cultures of adherent mouse bone marrow feeder cells were pre-

pared according to the method of Dexter et al. (1984). Fresh 4-week-old mouse bone marrow cells were plated in T-25 flasks (Nunc, Kamstrup, Denmark) in IMDM (Iscove's modified Dulbecco's medium) supplemented with 20% horse serum (Seromed, Batch no. S9135, Berlin, Germany) and antibiotics. They were incubated at 37°C, 7% CO<sub>2</sub> in a humidified atmosphere. The cultures were re-fed at day 3, changed at day 6, and used at day 10 after rinsing twice with IMDM. Mouse feeder layers were loaded with low-density, 3-week-old rat bone marrow cells separated by centrifugation (1000 g, 30 min) onto a 28% BSA cushion (Pentex BSA, Miles, IL, USA) as previously described (Deugnier et al., 1990). The number of nucleated rat bone marrow cells loaded per T-25 flask (3.5×10<sup>6</sup> cells) was estimated with a cell counting system (Coulter Counter ZM, Coultronics, FL, USA) by adjusting the cell diameter window between 5.38 µm and 11 µm. Cells were grown in IMDM containing 20% FCS (fetal calf serum; Biological Industries, Israel, batch no. 802255 and Seromed, Berlin, Germany, batch no. S0125) preabsorbed on charcoal (Norit A, Serva, Germany) and Dextran K70 (Pharmacia, Uppsala, Sweden), as described by Hayashi et al. (1984). This growth medium, referred to as "steroid-free conditions", was supplemented with 1% steroid-free synthetic serum (Ultrosor SF, IBF, Villeneuve-la Garenne, France) and antibiotics. In some specified experiments, 10<sup>-7</sup> M hydrocortisone succinate (Roussel, Paris, France) was added to cultures under steroid-free conditions. Cells were routinely grown for 3 days in 5% CO<sub>2</sub> at 37°C. At day 3, the non-adherent and the loosely adherent cells were harvested by gentle pipetting.

### Panning experiments

Cultured rat bone marrow cells were panned using antibody-mediated plate binding, as described (Takacs et al., 1988). Bacteriological Petri dishes (100 mm, Greiner, Labortechnik, Germany) were coated with 50 µg of goat anti-mouse Ig (ICN Biomedicals, High Wycombe, Bucks, UK), 5 ml of 10 µg/ml solution, in 0.05 M Tris buffer, pH 9.2, for 3 h at 23°C. Before use, plates were rinsed twice with PBS and incubated with PBS containing 5% FCS for 15 min. Fresh and cultured rat bone marrow cells (15×10<sup>6</sup> cells) were incubated either with HIS24 or with HIS50 mAbs diluted in 500 µl of cold IMDM containing 10% steroid-free FCS for 30 min at 4°C. Cells were washed with cold PBS, resuspended in 10 ml of cold IMDM plus 10% steroid-free FCS and poured onto two separate goat, anti-mouse IgG-coated plates. Plates were incubated at 4°C for 2 h. After incubation, non-adherent cells were removed by gently rinsing once with IMDM. Bound cells were gently scraped with a rubber policeman. Cells were washed twice before being counted and used for immunofluorescence labeling or subculturing in mouse thymic lobes.

### In vitro assay for rat thymopoiesis

The presence of T-cell precursors in fresh and cultured rat bone marrow cell populations was assessed by *in vitro* transfer into mouse fetal thymic organ cultures, as recently described (Deugnier et al., 1990). The thymic lobes were maintained in IMDM (GIBCO BRL, Cergy-Pontoise, France) supplemented with 10% FCS (Biological Industries, Israel, batch no. 802255 and Seromed, Berlin, Germany, batch no. S0125), 50 µM 2-mercaptoethanol, antibiotics and 1.35 mM 2-deoxyguanosine (dGuo, Sigma, MO, USA) for 7 days at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere. At day 7, each Nucleopore filter carrying 6 thymic lobes was transferred onto a new square of gelatin foam sponge in 35 mm Petri dishes containing 2 ml of fresh medium without dGuo. 2 µl of the suspension containing 10, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup> or 10<sup>5</sup> rat bone marrow cells in dGuo-free medium were loaded onto each thymic lobe. After 10-15 days in culture, the thymic lobes (12) loaded with the same bone marrow cell concentration were pooled and teased

between 2 squares of nylon sieve (mesh width of 100  $\mu$ m; Nytal, Switzerland) in IMDM. The number of harvested cells was estimated as described above. Comparable repopulations were obtained when using Balb/c and Swiss mouse thymic lobes. Mouse bone marrow used to establish stromal cell layers for rat lymphopoiesis and mouse thymic lobes used in rat thymopoiesis assays were taken from the same mouse strain.

### Cell immunolabeling and fluorescence analysis

Immunofluorescence surface labeling was done on live cells. For single labeling, cells ( $5\text{--}20 \times 10^4$  cells per well of microtiter plate) were first incubated for 1 h at 4°C with 30–50  $\mu$ l of the following mouse monoclonal antibodies: Ox-7 (anti-rat Thy-1.1), Ox-19 (anti-rat CD5), Ox-34 (anti-rat CD2), R7.3 (anti-rat TcR  $\alpha$ ), 1F4 (anti-rat CD3), Ox-22 (anti-rat L-CA restricted determinant), W3/13 (anti-rat leukosialin), HIS 24 and HIS50 (anti-rat pre-B-cell determinants), HIS 40 (anti-rat IgM) and Ox-42 (anti-rat CD11b/c, used as anti-myeloid determinant). Ox-7 (used at 1:100), Ox-34 (used at 1:100), Ox-42 (used at 1:100) and R7.3 (used at 1:1000) were obtained from Serotec (Oxford, UK). Ox-19, Ox-22 and W3/13 (used at 1:500) were kindly provided as ascites by Dr A. Williams (Medical Research Council, University of Oxford, UK). Anti-rat CD3 (used at 1:1000) was obtained from Dr T. Tanaka (Tohoku University, Sendai, Japan). HIS 24 and HIS 40 ascites were used at 1:100; HIS50 supernatant was used at 1:15. FITC-coupled, goat anti-mouse Ig (Nordic Immunology, Tilburg, NL), FITC-coupled donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) and PE-coupled, goat anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA, USA) were used as second antibodies (1:100, 30 min, 4°C). Controls were routinely done with second step reagent only. For CD3 staining, an isotype-matched mouse mAb (mouse IgM anti-quail neural crest cells, NC1) was used as a control (Vincent et al., 1983). For direct double-labeling (CD4/CD8), cells were incubated for 1 h at 4°C with a mixture of FITC-coupled, Ox-8 mAb (anti-rat CD8, used at 1:50) and PE-coupled, W3/25 mAb (anti-rat CD4, used at 1:5). FITC- and PE-conjugated mAbs were purchased from Serotec (Oxford, UK). For indirect double-labeling (HIS24/HIS50), determinants recognized by HIS24 and HIS50 were revealed with FITC-coupled, donkey anti-mouse IgG and PE-coupled, goat anti-mouse IgM respectively. After all staining procedures, live cells were fixed overnight in a 4% formaldehyde solution in PBS at 4°C and analysed by flow fluorometry with a FACScan (Becton Dickinson, CA, USA). Forward scatter was measured with linear amplification, whereas side scatter and fluorescence intensity were measured with logarithmic amplification. Data collected from  $3\text{--}6 \times 10^3$  cells were analysed with Lysis software in the form of dot plots and fluorescence histograms.

### In vitro GM-CFU assays

Fresh and cultured rat bone marrow cells ( $2 \times 10^4$  cells per 35 mm Petri dish; Greiner, Labortechnik, Germany) were cultured in 1 ml of IMDM made semi-solid with 0.9% (w/v) methylcellulose (Fluka, Switzerland) under steroid-free conditions. Hemopoietic growth factors were included at the following concentrations: 25 U/ml of pure recombinant interleukin 1 (rIL-1 from human monocytes, provided by Dr C. Damais, Roussel-Uclaf, France), 5 U/ml of recombinant rat IL3 (rIL-3 from serum-free medium conditioned by transfected Cos-1 monkey cells, provided by Dr A. Hapel, John Curtin School of Medical Research, Australia). A medium conditioned by adherent rat bone marrow stromal cells stimulated by rIL-1 (10 U/ml) for 48 h was added at 10%, as described by Keller et al. (1985). Semi-solid cultures were incubated for 10 days at 37°C in a humidified incubator with 7% CO<sub>2</sub> in air. Colonies (>50 cells) were counted with an inverted microscope.

### In vivo S-CFU assays

Groups of 7-week-old rats were anesthetized with pentobarbital (Sanofi, France) and irradiated with a therapeutic X-ray source (Philips 250kV) at a dose of 750 cGy (rate of about 20 cGy/min). One day later, the irradiated rats were re-anesthetized and injected intravenously with  $10^6$  fresh or cultured bone marrow cells. Rats were maintained for 12 days prior to killing. At the time of killing, the spleens were removed, fixed in Bouin's fixative and scored for the presence of macroscopic spleen colonies.

## RESULTS

### In vitro T-cell development from rat bone marrow precursor cells

We previously reported that Thy-1.1<sup>+</sup> rat bone marrow cells colonize dGuo-treated Thy-1.2<sup>+</sup> mouse fetal thymic lobes and undergo thymopoiesis giving rise to Thy-1.1<sup>+</sup> CD2<sup>+</sup> CD5<sup>+</sup> lymphoid cells, 75–80% consisting of CD4<sup>+</sup> CD8<sup>+</sup> TcR<sup>+</sup> cells, and 20–25% of both CD4<sup>+</sup> CD8<sup>+</sup> TcR<sup>low</sup> and CD4<sup>+</sup> CD8<sup>+</sup> TcR<sup>low</sup> cells (Deugnier et al., 1990). We have now extended the analysis of rat cells isolated from fetal thymic lobes after 14 days in vitro. As a cell source for fetal thymus repopulating assays, we used rat bone marrow enriched in hemopoietic precursor cells by centrifugation onto a BSA cushion, instead of total bone marrow. Using this low-density cell population, thymus reconstitution was reproducibly observed after loading  $10^4$  cells per thymic lobe. Flow cytometry analysis showed that a homogeneous lymphoid cell population developed after 14 days in vitro (Fig. 1A). These lymphoid cells were large blasts as compared to unseparated thymocytes (Fig. 1B). As illustrated in Fig. 1, mouse thymic lobes were almost fully repopulated with lymphoid cells of rat origin which expressed high levels of Thy-1.1 and CD2 identical to those of fresh thymocytes. Both cell populations were positive for CD43 and CD5, the lowest levels of expression characterizing thymus repopulating cells. These patterns are evocative of those obtained from immature CD5<sup>low</sup> CD4<sup>+</sup> CD8<sup>+</sup> CD3<sup>low</sup> thymocytes (Takacs et al., 1988). More than 90% of the developing cells were not stained by Ox-39 mAb which recognizes the p55 chain of the IL-2R characteristic of activated T cells (data not shown). On the other hand, the frequency of granulocyte macrophage colony-forming units (GM-CFU) in 14-day organ-cultured cells was less than 1 per  $10^5$  cells, as estimated in methylcellulose assays using total bone marrow and thymocytes as positive ( $133 \pm 22$  GM colonies per  $10^5$  cells) and negative (<1 GM colony per  $10^5$  cells) control cell populations.

Analysis of CD3 distribution patterns revealed the presence of about 30% CD3<sup>low</sup> and 10% CD3<sup>high</sup> cells after 14 days in organ culture (Fig. 1A). Similar results were obtained for TCR  $\alpha$ -expressing cells (data not shown). Although lower than on control thymocytes, CD3 expression on organ-cultured cells was significant, as shown by controls using isotype-matched mAb (Fig. 1A, B). We cannot exclude the possibility that in organ cultures CD3<sup>high</sup> cells arise from a contamination of resident bone marrow T cells. Nevertheless at day 14, the majority of organ-cultured cells displayed a Thy-1.1<sup>high</sup> CD2<sup>+</sup> CD43<sup>+</sup> CD5<sup>low</sup> CD3<sup>low</sup> blast cell phenotype, characteristic of developing

rat thymocytes (Paterson et al., 1987; Takacs et al., 1988; Tanaka et al., 1989). Moreover, significant CD3 and TcR expression only appeared between day 10 and day 14 (data

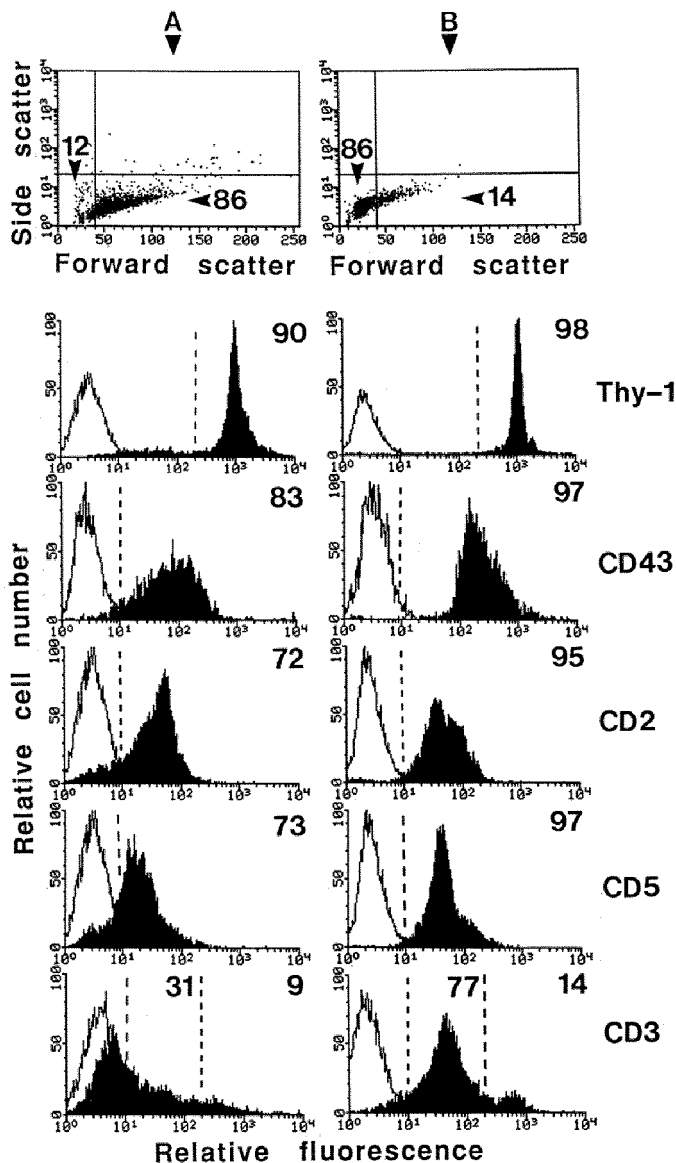
not shown), strongly suggesting that these characteristics were acquired in vitro in the thymus microenvironment.

#### Whitlock/Witte-type but not Dexter-type cultures of bone marrow contain unanchored thymus-repopulating cells

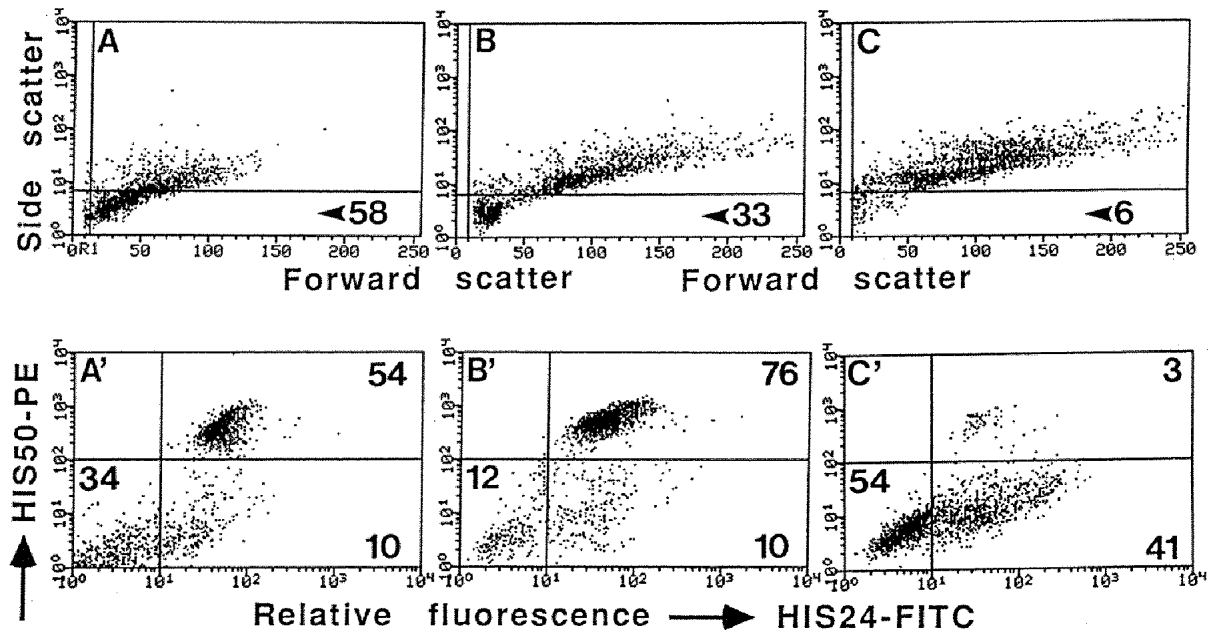
We investigated the thymus-repopulating activity of stroma-dependent, bone marrow cultures favouring either lymphopoiesis or myelopoiesis. Bone marrow cultures were initiated by loading low-density rat bone marrow cells onto pre-established mouse bone marrow feeder cells. The low-density cell population accounts for 10% of the unseparated bone marrow; it is depleted in mature erythroid and myeloid cells but is composed of approximately 60% lymphoid cells defined on the basis of their forward and side scatter properties (Fig. 2A). Double-labeling experiments showed that half of the lymphoid cell population coexpressed B lineage surface antigens revealed by HIS24 and HIS50 mAbs, while the other half was composed of HIS24<sup>+</sup> HIS50<sup>-</sup> and HIS24<sup>-</sup> HIS50<sup>-</sup> precursor cells (Fig. 2A'). HIS24 is the rat equivalent of mouse B220 (Opstelten et al., 1986) and HIS50 recognizes a determinant carried by pre-B and B cells in the rat bone marrow (Hermans et al., 1991). When grown under steroid-free conditions (Whitlock/Witte-type cultures) for 3 days, the low-density cells gave rise to a well-defined lymphoid cell population which accounted for 25-40% of the non-adherent cells and was enriched in HIS24<sup>+</sup> HIS50<sup>+</sup> cells (Fig. 2B and 2B'). In the absence of a pre-established stromal cell layer, the absolute number of lymphoid cells per flask was reduced at least by a factor of 2 and the lymphoid cell viability was highly compromised. Conversely, in the presence of 10<sup>-7</sup> M hydrocortisone (Dexter-type cultures) the lymphoid cell compartment was reduced to less than 10% of the unanchored cell fraction (Fig. 2C). Hydrocortisone clearly had a cytotoxic effect on HIS24<sup>+</sup> HIS50<sup>+</sup> B cells but spared HIS24<sup>+</sup> HIS50<sup>-</sup> and HIS24<sup>-</sup> HIS50<sup>-</sup> lymphoid precursor cells (Fig. 2C'). The number of cells capable of giving rise to GM colonies in methylcellulose assays was maintained throughout the 3-day culture period (data not shown).

Fetal thymic organ cultures were used to compare the thymus-repopulating activities of fresh low-density bone marrow cells, Whitlock/Witte-type bone marrow cultures and Dexter-type bone marrow cultures (Fig. 3). Between 50 to 80% of Thy-1.1<sup>high</sup> cells were obtained after loading fresh low-density cells or Whitlock/Witte-type cultures, whereas less than 20% of Thy-1.1<sup>high</sup> cells could be detected after initiating the organ cultures with Dexter-type bone marrow cultures (Fig. 3A). Accordingly, the development of Dexter-type cultured cells within thymic lobes was very poor, as compared to that of fresh low-density cells and Whitlock/Witte-type cultures (Fig. 3B). Loading 10 times more Dexter-type cultured cells per lobe did not modify the results (data not shown). All three cell populations gave rise intrathymically to, at most, 25% Thy-1.1<sup>low</sup> cells which have not yet been characterized.

Further analysis of the phenotype of rat bone marrow cells organ-cultured for 14 days showed that both fresh low-density cells and Whitlock/Witte-type cultures gave rise to Thy-1.1<sup>+</sup> CD2<sup>+</sup> CD43<sup>+</sup> CD5<sup>+</sup> cells with comparable percentages (Table 1). In both cases, the developing cell pop-



**Fig. 1.** Flow cytometry analyses of organ-cultured cells of bone marrow origin (lane A) and control thymocytes (lane B). Low-density Thy-1.1<sup>+</sup> rat bone marrow cells were organ-cultured in Thy-1.2<sup>+</sup> mouse thymic lobes at a concentration of 10<sup>4</sup> cells per lobe for 14 days. Representative results from two independent reconstitution experiments are presented. The upper panels show dot-plot displays of cells gated according to their forward and side scatter properties. Note that organ-cultured cells are predominantly large lymphoid cells, as compared to fresh thymocytes. Lower panels show fluorescence intensity distribution in lymphoid cell subsets after single cell labeling with the following mouse mAbs: Ox-7 (Thy-1.1), W3/13 (CD43), Ox-34 (CD2), Ox-19 (CD5) and 1F4 (CD3). Related controls stained with FITC-conjugated, goat anti-mouse Ig antibody only or with isotype-matched mAb followed by PE-conjugated, goat anti-mouse IgM (in the case of CD3 staining) appear as white histograms. In each panel, white and black histograms represent the analysis of five thousand cells.



**Fig. 2.** Flow cytometry analyses of fresh and cultured rat bone marrow cells. Upper panels show dot-plot displays of cells gated according to their forward and side scatter properties. Percentages of lymphoid cells versus total nucleated cells are indicated. Lower panels show two-color analysis of the gated lymphoid cell subsets stained with HIS24 and HIS50 mAbs, revealed by FITC-conjugated, donkey anti-mouse IgG and PE-conjugated, goat anti-mouse IgM antibodies, respectively. Relative percentages of double negative cells, single HIS24-positive cells and double positive cells are indicated. Double-stained cells correspond to rat pre-B and B cells. Low-density rat bone marrow cells were analysed as a fresh cell suspension (A, A'), and after a 3-day culture period on preestablished mouse bone marrow stroma either under steroid-free conditions (Whitlock/Witte-type culture; B and B') or in the presence of  $10^{-7}$  M hydrocortisone (Dexter-type culture; C and C'). Each panel represents the analysis of five thousand cells from a representative experiment of at least three studies.

ulation was made of about 30% CD3<sup>+</sup> cells, 15% CD8<sup>+</sup>CD4<sup>+</sup> cells and 15% CD8<sup>+</sup>CD4<sup>+</sup> cells. Single CD4<sup>+</sup> cells could not be detected. Late pre-B cells (HIS50<sup>+</sup> cells), B cells (sIgM<sup>+</sup>) and mature myeloid cells (CR3<sup>+</sup> cells) occurred at low frequency.

#### Short-term Whitlock/Witte-type cultures of bone marrow are enriched in thymus-repopulating cells but depleted in myeloid-restricted precursor cells

As reported in our previous study (Deugnier et al., 1990), optimal reconstitutions (defined on the basis of cell yield and positive assays) were obtained after loading  $10^5$  cells of total bone marrow or  $10^4$  cells from the low-density fraction per lobe. Similar dose-response assays show that cells from Whitlock/Witte-type cultures fully repopulated thymic lobes at an initial concentration of  $10^3$  cells per lobe, indicating a 100-fold enrichment in thymus-repopulating activity, as compared to the total bone marrow (Table 2). Subsequently, we reproducibly obtained full reconstitution after loading  $10^4$  cultured cells per lobe, so that a minimal 10-fold enrichment in thymus-repopulating activity could be obtained. Given the mean number of cells per flask collected at day 3 ( $2.2 \times 10^6 \pm 0.8 \times 10^6$  cells;  $n=10$ ) after initially loading  $3.5 \times 10^6$  cells, thymus-repopulating cells were at least maintained, if not amplified, during the 3-day culture period.

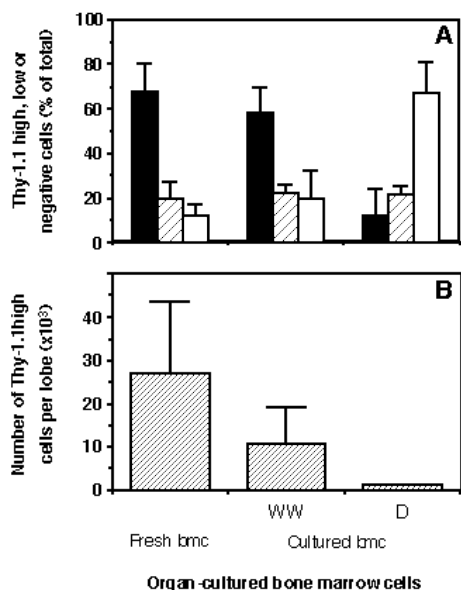
The number of cells recovered per day-14-reconstituted thymus was significantly higher when cells from the low-density fraction were used (Fig. 3B). Kinetics of thymus

repopulation indicated that this feature was observed from day 4 to day 14 in organ culture (Fig. 4), which demonstrates that precursor cells derived from fresh bone marrow and Whitlock/Witte-type cultures display parallel intrathymic development in organ culture.

The possible presence of myeloid-restricted precursor cells in our Whitlock/Witte-type cultures was then examined by performing *in vivo* spleen colony-forming unit (S-CFU) and *in vitro* GM-CFU assays (Fig. 5). Spleen colony-forming cells, at least 3 times enriched in the fresh low-density bone marrow fraction as compared to the total bone marrow, were markedly depleted after a 3-day culture period under steroid-free conditions (Fig. 5A). GM-CFU cells between day 3 and day 9 were detectable at a level that was consistently 20 times lower than that present at day 0 (Fig. 5B).

#### Thymus-repopulating activity of Whitlock/Witte-type cultures is predominantly associated with the HIS24<sup>+</sup> lymphoid cell subset

As illustrated above (Fig. 2B), the unanchored fraction of Whitlock/Witte-type cultures of bone marrow included a distinct lymphoid cell subset, mostly composed of HIS24<sup>+</sup> HIS50<sup>+</sup> pre-B and B cells. It also contained mature myeloid cells (essentially macrophages), which is a common feature of bone marrow culture development (Kincade et al., 1989). Flow cytometry analysis, after exclusion of the myeloid cells on the basis of their side scatter properties, showed that the lymphoid cell subset was reproducibly composed



**Fig. 3.** Fetal thymic organ cultures of fresh and cultured rat bone marrow cells depicted in Fig. 2. Low-density rat bone marrow cells taken as a fresh cell suspension (fresh bmc) and after a 3-day culture period on preestablished mouse bone marrow stroma, either under steroid-free conditions (Whitlock/Witte-type culture; WW), or in the presence of  $10^{-7}$  mM hydrocortisone (Dexter-type culture; D), were organ-cultured for 15 days. Individual mouse thymic lobes were initially loaded at a concentration of  $10^4$  rat cells per lobe. Organ-cultured cells were collected and counted before being labeled with Ox-7 mAb (Thy-1.1). Results are shown as (A) percentages of rat donor Thy-1.1<sup>high</sup> cells (black column), Thy-1.1<sup>low</sup> cells (hatched column) and percentages of Thy-1.1<sup>neg</sup> cells (white column) in reconstituted thymic lobes, and (B) absolute number of rat donor Thy-1.1<sup>high</sup> cells per lobe. Data are expressed as mean  $\pm$  s.d. of 3 independent experiments, except in the case of Dexter-type cultures which gave rise to a non-significant number of Thy-1.1<sup>high</sup> cells ( $< 20$  cells per lobe) in 1 of 3 reconstitution assays.

of small ( $80 \pm 6$ ;  $n=5$ ) and large ( $20 \pm 6$ ;  $n=5$ ) lymphoid cells (Fig. 6). The small lymphoid cell subset was clearly enriched in HIS24<sup>+</sup> HIS50<sup>+</sup> pre-B and B cells, as compared to the large cell compartment which contained approximately 20% of HIS24<sup>+</sup> HIS50<sup>+</sup> and 40% of HIS24<sup>+</sup> HIS50<sup>+</sup> lymphoid precursor cells.

HIS24<sup>+</sup>, HIS24<sup>+</sup> and HIS50<sup>+</sup> cell populations enriched by panning were tested for their ability to repopulate thymic lobes in comparison with the unseparated cultured cells (Table 2). These experiments showed that the thymus-repopulating cells were concentrated in the HIS24<sup>+</sup> cell subpopulation and absent from the HIS 50<sup>+</sup> cell subset, which is consistent with studies reporting that mouse thymus-repopulating cells are found in a minor subset of Thy-1<sup>low</sup> B220<sup>+</sup> cells from the fresh bone marrow (Spangrude et al., 1988; Ikuta et al., 1990; Palacios et al., 1990). As few as 100 HIS24<sup>+</sup> cells were able to develop in organ culture, giving rise to Thy-1<sup>high</sup> cells; like unseparated cultured cells, HIS24<sup>+</sup> cells produced CD4<sup>+</sup> CD8<sup>+</sup> cells and CD4<sup>+</sup> CD8<sup>+</sup> cells intrathymically (data not shown). Enriched HIS 24<sup>+</sup> cells which contained about 10% HIS50<sup>+</sup> lymphoid cells (Fig 2A') also appeared to be capable of repopulating thymic lobes. Nevertheless, as panning efficiency for pos-

**Table 1.** Phenotype of lymphoid cells developing in organ-cultured thymic lobes from fresh and cultured rat bone marrow cells

Antigen	Antibody	Cell source used in organ culture	
		Low-density cells	W/W cultured cells
Thy-1.1	Ox-7	69 $\pm$ 7	75 $\pm$ 11
		88 $\pm$ 6*	82 $\pm$ 5*
CD43	W3/13	68 $\pm$ 7	73 $\pm$ 16
CD5	Ox-19	51 $\pm$ 8	68 $\pm$ 11
CD2	Ox-34	54 $\pm$ 12	75 $\pm$ 8
CD3 low	1F4	20 $\pm$ 5*	23*
CD3 high	1F4	8 $\pm$ 3*	8*
CD8	Ox-8	14 $\pm$ 9	14 $\pm$ 4
CD4/CD8	Ox-8+W3/25	13 $\pm$ 6	15 $\pm$ 5
–	HIS 50	13 $\pm$ 9	4
sIgM	HIS 40	1 $\pm$ 1	3.5
CR3	Ox-42	2 $\pm$ 2	9

Individual mouse thymic lobes were loaded with  $10^4$  low-density rat bone marrow cells, taken as a fresh cell suspension or after a 3-day culture period on preestablished stroma under Whitlock/Witte conditions (W/W cultured cells). At day 14, organ-cultured cells were labeled with the mAbs indicated and analysed by one- or two-color flow cytometry, as described in Fig. 1. Unspecific labeling is deducted from the present values. Results are shown as mean percentages of positive cells ( $\pm$  s.d.) from 2 to 3 separate reconstitution assays.

\*Data obtained in a second group of comparative assays.

**Table 2.** Thymus-repopulating activities of precursor cells from Whitlock/Witte-type cultures of bone marrow

Cell source	Thymic lobe reconstitution	
	Assays	Number of Thy-1 <sup>high</sup> cells in positive assays
10 <sup>3</sup> cells per lobe		
Unseparated cells	+/+	8477
HIS 24 <sup>+</sup> cells	+/+	13234
HIS 24 <sup>+</sup> cells	+/-	1695
HIS 50 <sup>+</sup> cells	-/-	ND*
10 <sup>2</sup> cells per lobe		
Unseparated cells	+/-	563
HIS 24 <sup>+</sup> cells	+/+	2786
HIS 24 <sup>+</sup> cells	-/-	ND*

Unseparated 3-day Whitlock/Witte-type cultured cells and cultured cells enriched in HIS24<sup>+</sup>, HIS24<sup>+</sup> and HIS50<sup>+</sup> cell subsets by the panning method were loaded onto individual thymic lobes at the cell concentration indicated. After 14 days, organ-cultured cells were collected, counted and labeled with Ox-7 mAb before FCM analysis. For each cell subset, two to three independent assays were performed, each comprising 12 pooled thymic lobes. Positive assays (in which a thymic lobe contained  $> 5 \times 10^3$  cells) are quoted as +. Results are also shown as mean number of Thy-1.1<sup>high</sup> cells per lobe in positive assays.

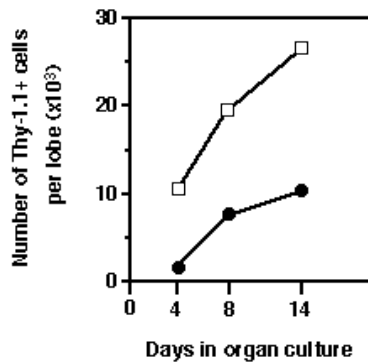
\*Not determined, because of too few cells per assay.

itively selected cells was estimated to be equal to 60%, contamination by HIS24<sup>+</sup> cells may account for the reconstitution observed with  $10^3$  HIS24<sup>+</sup> cells per lobe. In contrast, negatively selected cells were 90% pure, as indicated by flow cytometry analysis of HIS24<sup>+</sup> panned cells (data not shown).

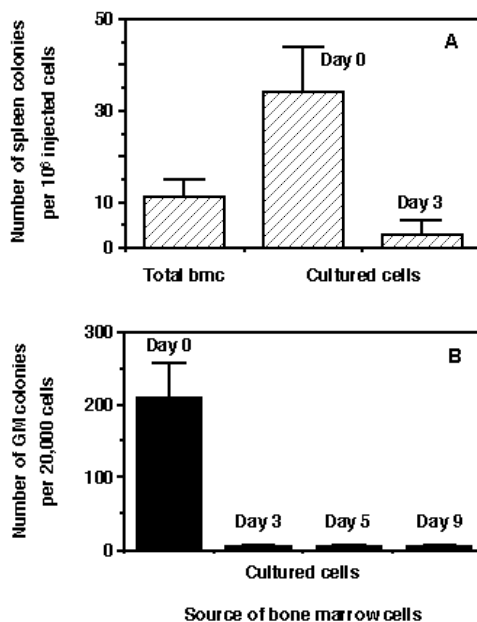
## DISCUSSION

We report here that short-term cultures of bone marrow, grown on pre-established stromal cells under steroid-free

conditions, sustain lymphopoiesis, block myeloid-restricted precursor cell production and are significantly enriched in unanchored lymphoid precursor cells capable of initiating thymopoiesis in fetal thymic organ cultures. Thymus-repopulating activity from bone marrow cultures is predomi-



**Fig. 4.** Time course of thymic lobe repopulation using low-density bone marrow cells (□) and 3-day Whitlock/Witte-type cultures of bone marrow (●). Cells were concomitantly loaded at an initial concentration of  $10^4$  cells per lobe; they were analysed for Thy-1.1 expression as described in Fig. 3 after 4, 8 and 14 days in organ culture. Results are from a single thymus repopulation experiment.



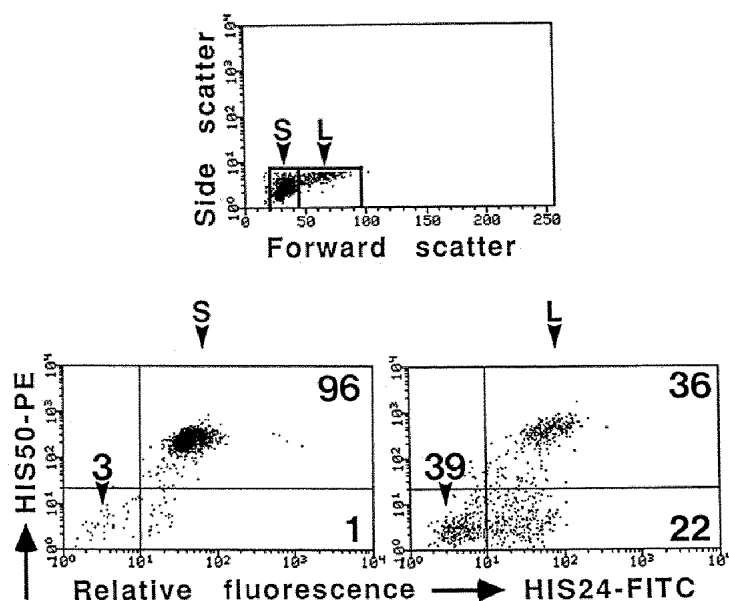
**Fig. 5.** Myeloid-restricted precursor cell activity of short-term Whitlock/Witte-type cultures of bone marrow as compared to fresh bone marrow. (A) In vivo spleen colony formation of unseparated bone marrow cells (bmc), low-density bone marrow cells before (day 0) and after a 3-day culture period under Whitlock/Witte conditions (day 3). (B) In vitro GM colony formation of low-density bone marrow cells before and after being cultured under Whitlock/Witte conditions for 3, 5 and 9 days. S-CFU and GM-CFU assays were performed as described in Materials and methods. For S-CFU assays, results are expressed as mean ( $\pm$  s.d.) of 4 to 5 animals injected in 2 independent assays. For GM-CFU assays, data are shown as mean ( $\pm$  s.d.) of triplicate semi-solid culture dishes.

nantly associated with a 10% cell subset of steroid-sensitive, HIS24<sup>-</sup> lymphoid precursors.

Rat T-cell development from fresh and cultured bone marrow cells was followed in an in vitro mouse thymus repopulation assay by means of phenotypic analysis. Unlike in vivo experimentations after intrathymic transfer, in vitro repopulation of thymic lobes implies an active cell migration step which partly mimics thymic selection of bone marrow precursor cells transferred intravenously. In the mouse system, this organ culture method has proved to be very useful for manipulating limited numbers of precursor cells of intrathymic and extrathymic origin, while allowing the recovery of almost pure populations of donor-type thymocytes identified by their Thy-1 expression (Ikuta et al., 1990; Sharp et al., 1990; Kingston et al., 1985; Liu and Auerbach, 1991). In agreement with these studies, we found that the microenvironment of dGuo-treated fetal Thy-1.2 mouse thymic lobes selectively supports the development of Thy-1.1<sup>high</sup> rat lymphoid cells of bone marrow origin which did not display myeloid precursor activity. Both fresh low-density cells and Whitlock/Witte-type cultures loaded at an initial cell concentration of  $10^4$  cells per lobe gave rise intrathymically to appreciable numbers of Thy-1.1<sup>high</sup> cells (between  $5 \times 10^3$  and  $50 \times 10^3$  cells per lobe), accounting for up to 90% of the organ-cultured cells at day 14. As Thy-1.1 is not present on resting mature T cells or on NK cells in the rat but is expressed on immature bone marrow lymphoid cells (including B-lineage cells), on thymocytes and on a small subset of activated T cells (Paterson et al., 1987; Takacs et al., 1988; Opstelten et al., 1986; Hermans, 1991; van den Brink et al., 1990), in vitro thymopoiesis was also investigated by a panel of T- and B-lineage-specific markers. The phenotypic profiles obtained strongly support the notion that repopulation of fetal mouse thymic lobes by rat Thy-1.1<sup>high</sup> cells of bone marrow origin can be used as a readout assay for initiation of thymopoiesis from bone marrow CD3<sup>-</sup> TCR<sup>-</sup> precursor cells. Such a rat-mouse chimera is physiologically relevant, since two independent studies recently demonstrated the stable, long-term engraftment of rat fetal liver and bone marrow cells in irradiated SCID and normal mouse recipients (Ildstad et al., 1991; Surh and Sprent, 1991). Our preliminary experiments, using SCID mice as recipients for rat bone marrow low-density cells, indicate that a full spectrum of rat thymocytes (including 80% CD4<sup>+</sup>CD8<sup>+</sup>, 8% CD4<sup>+</sup>CD8<sup>-</sup> and 7% CD4<sup>-</sup>CD8<sup>+</sup> cells) can be detected in the host thymus 3 weeks after intravenous injection of the rat cells.

In agreement with the previous study reporting co-enrichment of thymus-repopulating cells and myeloid-restricted precursor cells in mouse bone marrow (Spangrude et al., 1988; Ikuta et al., 1992), we found that low-density rat bone marrow cells were 10-fold enriched in thymus-repopulating cells and at least 3 times enriched in day 12 S-CFU as compared to the total bone marrow. However, after a 3 day culture period under steroid-free conditions, the enrichment in thymus-repopulating cells was maintained while the frequency of myeloid-restricted precursor cells was dramatically reduced. The frequency of S-CFU can be calculated to be one per  $3 \times 10^5$  cells, that is one per  $0.75 \times 10^5$  cells of the lymphoid cell subpopulation, which accounts for at least 25% of the cultured bone marrow. In the same type of cul-





**Fig. 6.** Flow cytometry analysis of gated lymphoid cells from 3-day Whitlock/Witte-type cultures of rat bone marrow. The upper panel, representing the analysis of six thousand cells, shows dot-plot displays of lymphoid cells gated according to their forward scatter properties. Small (S) and large (L) lymphoid cell subsets are indicated; they account for 84% and 16% of the total lymphoid cell subset, respectively. Lower panels show two-color cytofluorometric analyses of the small and large lymphoid cell subsets after cell staining with HIS24 and HIS50 mAbs as described in Fig. 2. Relative percentages of double negative cells, single HIS24-positive cells and double positive cells are indicated. Note that immature HIS24<sup>+</sup> cells are concentrated in the large lymphoid cell subpopulation.

tures, the minimal frequency of thymus-repopulating cells can be estimated to be 1 in 250 lymphoid cells, putting forth a frequency of one thymus-repopulating cell per  $10^4$  total bone marrow nucleated cells, as discussed in our previous report (Deugnier et al., 1990). Thus short-term Whitlock/Witte-type cultures of bone marrow appear to be extremely efficient in functionally separating distinct cell subsets such as thymus-repopulating cells from myeloid-restricted precursor cells. These cell subsets are not yet distinguishable by a specific set of surface markers (Ikuta et al., 1992; Palacios et al., 1990).

Our data show that fresh low-density cells and Whitlock/Witte-type cultures, but not Dexter-type cultures, were able to repopulate thymic lobes *in vitro*. Moreover, in positive repopulation assays, the cell yield was dependent on the source of thymus-colonizing cells. Although predominantly composed of developing thymocytes, reconstituted thymic lobes contained discrete subpopulations of B cells and myeloid cells. Besides thymic epithelial cells, these accessory cells of bone marrow origin, which are also present *in vivo* (Surh and Sprent, 1991; van Ewijk, 1991), may interfere with the *in vitro* development of T-cell precursors. Thus, the higher intrathymic growth potential of low-density cells, as compared to cells from Whitlock/Witte-type cultures, may either be linked to intrinsic properties of precursor cells or to the effects of certain subsets of accessory cells, or to both. We favour the first hypothesis, since bone marrow cells depleted or not in adherent accessory cells displayed similar capacities for thymus repopulation (data not shown). On the other hand, the poor thymus-repopulating activity of our Dexter-type cultures is compatible with the notion that the unanchored cell populations spared by hydrocortisone are mainly composed of myeloid progenitor cells (Dexter et al., 1984). Nevertheless, cells reconstituting T-cell function in mice have been reported to be associated with the adherent stromal cell layer of Dexter-type cultures. These cells could be lymphoid-restricted stem cells (Fulop and Phillips, 1989) or lympho-myeloid stem cells (Fraser et al., 1990). Thus, in addition to steroid-resis-

tant T-cell precursors, our data may imply the existence of a steroid-sensitive bone marrow cell subset capable of thymus repopulation. *In vivo* experiments are currently being carried out to define the homing properties of this cell subset.

Very recently, two independent studies indicated that the intrathymic CD4<sup>+</sup> CD8<sup>+</sup> CD3<sup>+</sup> pre-T-cell population displays no myeloid precursor cell activity but contains cells capable of giving rise to B cells and NK cells *in vivo* (Wu et al., 1991; Rodewald et al., 1992). These results support the view that the thymus could be colonized by lymphoid-restricted precursor cells rather than by multipotential precursor cells. Consistently, our study strongly suggests that the HIS24<sup>+</sup> thymus-repopulating cells of Whitlock/Witte-type cultures are committed lymphoid cells. Firstly, these cells are enriched in culture conditions which promote the development of pre-B and pre-NK cells but not the maintenance of myeloid-restricted precursor cells (this study and Dorshkind, 1990; Hayashi et al., 1984; van den Brink et al., 1990). Secondly, they are found in the unanchored fraction of the cultures. Cell adhesion to bone marrow stromal cells is related to the differentiation program of hemopoietic cells in long-term bone marrow cultures, as well as in short-term adhesion assays (Coulombel et al., 1983; Dexter et al., 1984; Miyake et al., 1991; Verfaillie et al., 1990; Siczowski et al., 1992). Primitive precursor cells with growth potential and marrow-repopulating ability are preferentially associated with the adherent cell layer, which is composed of a heterogeneous bone marrow-derived stromal cell population producing extracellular matrix components. In contrast, the unanchored cell fraction mostly contains committed cells similar to those that are released from the bone marrow *in vivo*. Interestingly, a bone-derived mesenchymal precursor cell line of rat origin was found to support rat bone marrow cell binding and short-term lymphopoiesis (Z. Prakapas et al., *in preparation*), which allows us to further investigate the adhesion mechanisms of rat thymus-repopulating cells in the marrow environment. Such an approach should help to better define thymus-homing

cells and provide insights into the molecular events controlling T-cell precursor extravasation from the bone marrow.

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